(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 440 219 A1**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number: **91101278.9**

(22) Date of filing: **31.01.91**

(51) Int. Cl.5: **C12N 15/45**, C12N 15/47, C12N 5/10, A61K 39/165, A61K 39/205, C12N 7/00

(30) Priority: **02.02.90 EP 90102120**

(43) Date of publication of application:
07.08.91 Bulletin 91/32

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(71) Applicant: **SCHWEIZ. SERUM- & IMPFINSTITUT BERN**
Postfach 2707
CH-3001 Bern(CH)

(72) Inventor: **Billeter, Martin A., Prof. Dr.**
Balgriststrasse 70
CH-8008 Zürich(CH)
Inventor: **Cattaneo, Roberto**
Schauenbergstrasse 29
CH-8046 Zürich(CH)
Inventor: **Schmid, Anita**
Allenmoosstrasse 123
CH-8057 Zürich(CH)
Inventor: **Ballart, Isidro**
Kreuzwiesen 12
CH-8050 Zürich(CH)
Inventor: **Eschle, Daniel**
Leonhardhalde 15
CH-8001 Zürich(CH)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4 P.O. Box 86 07 67
W-8000 München 86(DE)

(54) **cDNA corresponding to the genome of negative-strand RNA viruses, and process for the production of infectious negative-strand RNA viruses.**

(57) The present invention relates, in general, to a methodology suitable for generating infectious negative-stranded viruses, suitable for use as vaccines, from cloned complementary deoxyribonucleic acid (cDNA). The invention relates specifically to cDNA molecules and to helper cells suitable as tools in this methodology and to the characterization of the resulting viruses. Measles virus (MV) is used as a model for negative-strand RNA viruses. The described invention provides the technology for construction of recombinant MV vaccine strains encoding epitopes or entire proteins from heterologous viruses, bacteria, or parasites. With slight modification of the technology described, any negative-stranded RNA virus can by used as vector in place of MV.

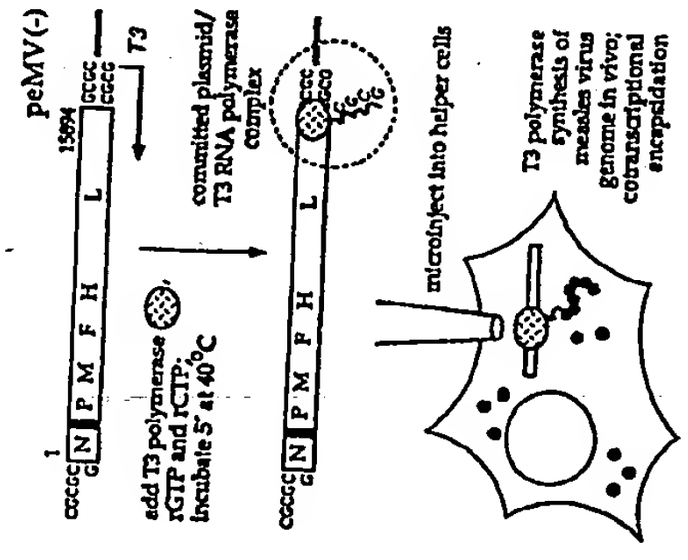

Figure 3

EP 0 440 219 A1

CDNA CORRESPONDING TO THE GENOME OF NEGATIVE-STRAND RNA VIRUSES, AND PROCESS FOR THE PRODUCTION OF INFECTIOUS NEGATIVE-STRAND RNA VIRUSES

The present invention relates, in general, to a methodology suitable for generating infectious negative-strand RNA viruses, suitable for use as vaccines, from cloned complementary deoxyribonucleic acid (cDNA). The invention also relates to cDNA molecules suitable as tools in this methodology. Measles virus (MV) is used as a model for negative-strand RNA viruses. The described invention provides the technology for construction of recombinant MV vaccine strains encoding epitopes or entire proteins from heterologous viruses, bacteria or parasites. The method described hereafter can be also applied in principle to any other negative-strand RNA virus instead of MV.

MV is a member of the paramyxovirus family. Its genetic information is encoded on a single strand of RNA (~$5x10^6$ daltons) with negative polarity. The genome is sequentially transcribed from the 3' terminus to yield 6 major separate ribonucleic acid (RNA) species, each of which encodes one major protein. The genome map is shown in Figure 2 (top), indicating the genes encoding these main products: N (nucleocapsid), P (phosphoprotein), M (matrix), F (fusion), H (hemagglutinin) and L ("large" = polymerase). Several additional RNA and protein species complicate this simple picture, but they are not particularly relevant here.

MV is a major cause of acute febrile illness in infants and young children. At present, several live attenuated MV vaccine strains are used (including Edmonston A and B and their derivatives, the Schwarz and Edmonston-Zagreb strains). Measles vaccine is usually administrated at 15 months of age in developed countries and at 4 to 6 months in developing areas; it has been shown to be highly effective in preventing disease. However, the genetic alterations responsible for attenuation of these vaccine strains remain unknown. The proven safety of measles vaccine, combined with the fact that it is routinely administrated to young children, makes it an ideal "carrier" for heterologous genes. Such a vaccine may prove useful in eliciting immune protection against other pathogenic agents in addition to the protection against the "carrier" virus itself.

The study of the replication cycle of a number of RNA viruses has been greatly facilitated by the availability of DNA clones from which infectious virus can be derived. Initially the Qβ bacteriophage, then poliovirus and other picornaviruses, and more recently Sindbis, another plus-strand animal RNA virus, were expressed from cloned cDNA (Taniguchi et al, 1978; Racaniello and Baltimore, 1981; Rice et al, 1987). Infectious clones of several plus strand plant RNA viruses (Ahlquist et al, 1984; Dawson et al., 1986; Meshi et al., 1986), an insect plus strand RNA virus (Dasmahapatra et al., 1986), and several viroids and plant satellite RNAs were also described (Cress et al., 1983; Tabler and Sänger, 1985; for references see Simon et al., 1988), and proviral DNA of retroviruses is infectious. Several engineered plus strand RNA viruses have been described for potential use as vectors for immunization purposes, eg. poliovirus, (Evans et al.,1989) and Sindbis (Xiong et al., 1989). However, attempts to obtain infectious virus from cDNA clones of negative-strand RNA viruses have failed so far. This is due to two properties of these viruses: (i) neither genomic nor antigenomic RNAs are infectious, because they do not serve as mRNAs; and (ii) both transcription and replication require ribonucleocapsids, i.e., helical nucleoprotein complexes, containing the genomic RNA and several proteins with structural or enzymatic function. In the case of MV, ribonucleocapsid consists of a ~ 16 kb long genomic RNA and at least three proteins, the structural nucleocapsid (N) protein, a polymerase (L for large), and a polymerase cofactor (P for phosphoprotein).

In the past, a variety of DNA viruses and positive-strand RNA viruses have been used as carriers to direct the expression of heterologous genes or gene segments in host cells, with the aim to elicit immune protection against the pathogen from which the heterologous genetic material was derived. In addition to the RNA viruses cited above, the DNA viruses vaccinia virus (Niacin et al., 1982; Weir et al.), cytomegalovirus (Spaete and Mocarski, 1987) and herpes virus (Spaete and Frenkel) should be mentioned here as examples. However, the use of such viruses as carriers suffers from several drawbacks including: (i) potential for inducing severe adverse reactions, as in the case of vaccinia virus; (ii) difficulty in genetic manipulation, specifically limitations on the size of the foreign genetic material which can be introduced into the viral genome without prohibiting packaging in the virion, as in the case of polio virus.

Recently Luytjes et al. (1989) have described a procedure by which a single gene of influenza virus (a segmented negativ-strand RNA virus) can be manipulated recombinant DNA techniques and then amplified in a virus-dependent fashion. In this case, a recombinant RNA containing a foreign coding sequence (chloramphenicol acetyltransferase flanked by the terminal sequences of the viral NS gene) is derived in vitro from plasmid DNA. This RNA is then covered with purified influenza A virus protein and introduced into cells superinfected with helper virus, leading to formation of viral particles which contain not only the 8

in a first step the short plasmids pePMF2 and peFHL, respectively, cleaved at their unique SpeI restriction sites, were used to introduce the chip constituting the framework for the additional transcription unit. The chip was assembled (by annealing) from four synthetic oligonucleotides (positive strand elements, 36 and 46 nucleotides and negative strand elements, 40 and 42 nucleotides; junction sites indicated by arrowheads in Figure 9, bottom). The protruding single-stranded regions on both sides of the chip fit into the 5' overhangs of the SpeI recognition site; ligation of the chip into the plasmids in the desired orientation leads to the recreation of a SpeI cleavage recognition site at the righthand border of the chip, in contrast to the lefthand border, where only a sequence containing 5 of the 6 nucleotides required for SpeI recognition is formed. This allowed easy selection of the plasmids containing the chip in the desired orientation; the expected sequence in and around the chip was confirmed by sequence determination. In a second step, two plasmids containing the complete MV cDNA with chips inserted in the 3' terminal region of either gene P (peMV(-)chipP) or gene H (peMV(-)chipH) were generated by three-fragment-ligations. A long acceptor fragment obtained by cleavage with SacII and SpeI from plasmid peMV(-) (involving a short 3' terminal portion of gene H, the entire L gene, the modified bluescript vector sequence linking the two extremities of the genomic MV cDNA insert, the entire N and a 5' terminal portion of the P gene) was used for both constructs. Two fragments spanning the region between SacII and NarI sites, recovered from pePMF2 or its derivative pePMF2chip, and NarI - SpeI fragments from peFHL or its derivative peFHLchip, in the appropriate combinations, were ligated to the acceptor fragment.

The final constructs were shown with our helper cell system to give rise to replicating MV yielding normal titers of infectious virions. As for the other reconstructed viruses described above, it was important to ascertain whether the rescued virus genomes agreed with the expected sequences. For this purpose, we tested by PCR amplification whether an RNA segment according to the chip inserted into the plasmids was still present in the RNA genomes and their complementary RNA involved in genome replication. Therefore, RNA was isolated from cells infected with the recovered viruses and reverse transcriptase reactions were primed with oligonucleotides of positive and negative polarity, mapping 50 - 100 nucleotides upstream and downstream, respectively of the regions of interest. The reverse transcriptions were followed by 25 cycles of PCR amplification, using the same oligonucleotides as for reverse transcription. The PCR reaction products around the P/M boundary were expected to be 167 nucleotides long in case of the unmodified region and 249 nucleotides in case of the inserted chip; for the H/L boundary the respective values were 131 and 213 nucleotides. As comparison for the PCR product length, DNA of the two constructed plasmids giving rise to the viruses was used as template. Figure 10 shows the analysis of the PCR products by agarose gel electrophoresis, confirming that the PCR products derived from MV genomic RNA templates are of identical size as the products derived from the plasmid templates.

Thus, the reconstructed viruses can support the insertion of a segment of 82 nucleotides in the indicated two positions of the genome, apparently without compromizing viral viability.

The present invention demonstrates that cloned DNA corresponding to the genome of a negative strand RNA virus can give rise to a replicating virus. Infectious virus can be recovered from cells microinjected with plasmids which generate either the MV genome or the antigenome. However, only few plaques were recovered from the supernatant of helper cells. Trivial reasons for the initial low production of infectious virus may be the damage of many of the 100-200 plasmid/polymerase complexes injected per cell either by the microinjection procedure or the action of cellular nucleases or proteases. In addition, MV genomes containing 2-5 additional nucleotides at each end likely require trimming *in vivo* to the precise genomic length before starting productive replication, as seems to the case for all the infectious RNAs examined until now (Gilvarg et al., 1975; Taniguchi et al., 1978; Ahlquist et al., 1984; Dawson et al., 1986; Meshi et al., 1986; Dasmahapatra et al., 1986; Rice et al., 1987). Most importantly, the presence of nonfunctional envelope proteins in helper cells could prevent efficient formation of infectious viral particles, since the envelope of virus particles budding from microinjected IP-3-Ca cells probably contains a mixture of functional M, F and H proteins, produced *de novo* from copies of the microinjected MV genome, and nonfunctional proteins, present in relatively high abundance both within as on the surface of helper cells. Furthermore, many of the budding virus particles probably enclose defective IP-3-Ca genomes. Only virions containing both a sufficient amount of functional envelope proteins and an Edmonston-Re genome, will be able to interact with the receptors on Vero cells, to fuse their envelope with the cellular membrane, and then to start lytic infection.

As expected, after two passages in Vero cells, reconstituted viruses generated titers by orders of magnitude higher than after microinjection, but not significantly lower than those reached by the Edmonston strain. Analysis of the 5' ends of the genome and antigenome of these reconstituted virus by primer extension revealed that these ends were identical to those of the Edmonston strain virus, confirming that the additional nucleotides flanking the genomic sequence in the primary transcript from the cloned cDNA are

removed precisely in vivo by a mechanism not known so far, as it has shown to be the case for all positive-strand RNA viruses expressed from cloned cDNA.

The establishment of a replication system based on cloned cDNA is particularly important for MV because it will now be possible to use recombinant DNA technology in the study of a virus for which only rudimentary genetics and *in vitro* transcription/replication systems are available (Rager-Zisman et al., 1984; Ray and Fujinami, 1987). The development of similar systems will be of great advantage in the study of other nonsegmented, negative strand RNA viruses of medical relevance, such as mumps virus, respiratory syncytial virus, human parainfluenza viruses or rabies virus. For some of these viruses, cell lines infected with conditionally lethal mutants could provide helper function. For other viruses, helper cell lines in which functional genes or synthetic mRNAs have been introduced could be used.

Infectious full length or defective RNAs produced from cDNA clones as described here will also open new experimental possibilities for the study of model negative-strand RNA viruses such as vesicular stomatitis virus or Sendai virus, when used in combination with existing complementation systems (Chattopadhyay and Banerjee, 1987; Meier et al., 1987; Whitt et al., 1989; Gotoh et al., 1989). Even segmented negative-strand RNA viruses like influenza can be rescued by the method of the present invention, using all eight genome segment sequences in the form of cloned cDNAs as described herein for MV.

MATERIALS AND METHODS

Cells and viruses

The Edmonston vaccine MV strain was propagated in HeLa (S3) suspension cell cultures and titered on Vero cell monolayers, basically as described (Udem, 1984). The SSPE-derived IP-3-Ca cell line (Burnstein et al., 1974), was propagated in monolayers essentially as detailed previously (Sheppard et al., 1986).

Transcription and microinjection

BssHII-linearized plasmids peMV(-) or peMV(+) were incubated for 5 minutes at 40°C with T3 polymerase (Genofit, Geneva, Switzerland) or at 37°C with T7 RNA polymerase (Pharmacia, Uppsala, Sweden) in a buffer containing 40 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.1mM GTP and 0.1 mM CTP, but without ATP and UTP, to stop RNA synthesis after 4 and 2 nucleotides, respectively. 1,4-dithio-DL-threitol, spermidine and RNAsin were excluded to avoid potential cell toxicity. The plasmid/phage polymerase complex was then diluted 1 to 5 in 0.15 M KCl and microinjected into the cytoplasm of 100-150 cells in a lawn of IP-3-Ca cells grown on coverslips, using Microinjector 5242 (Eppendorf, Hamburg, FRG). The needles were prepared with a Mecanex needle puller (Grade S.A., Nyon, Switzerland), using glass capillaries of 1.2 mm diameter (Clark Electromedical Instruments, Pangbourne, UK). Injection times and pressures were adjusted to deliver ~0.5 pl solution, containing 100-200 molecules of plasmid/phage polymerase complex. After injection, IP-3-Ca cells were cultivated for up to 14 days, supernatants were collected at different time points and stored at -70°C until they were analysed for infectivity in a plaque assay on Vero cell monolayers.

RNA extraction, reverse transcription, polymerase chain reaction (PCR) and sequencing

RNA was extracted from infected Hela S3, Vero or IP-3-Ca cells using a modified lithium chloride-urea protocol (Auffray and Rougeon, 1980; Cattaneo et al., 1984). Total RNA (2.5 µg) was used for reverse transcription, by priming with synthetic oligonucleotides specific for the 3' end of the N and P mRNAs as described (Schmid et al., 1987). One fifth of the cDNA recovered was amplified directly by PCR with Taq polymerase (Saiki et al., 1988) using the following primers: for the N gene
5'-(862)GAAATGATATGTGACATTGATAC(884, + strand), and
5'-(1741)TTATAACAATGATGGAGGGTAGGCG (1717, - strand). For the P gene:
5'-(1747)(CG)TTAGGAACCAGGTCCACAGAG (1767, + strand), and
5'-(3399)TTATAATGGATTTAGGTTGTACTAGTTG (3372, - strand). Nucleotides in parenthesis are not colinear with the MV genome; positions are indicated as in Cattaneo et al. (1989b), EMBL/Genbank accession number X 16565-9. Cycles of 1'94°C, 2'50°C and 10' 70°C were repeated 30 times. The amplified fragments were purified on a low melting agarose gel and extracted with Gene Clean (Bio 101, La Jolla, California). Sequencing with two of the primers described above, Sequenase and ^{35}S-labelled nucleotides was done according to a modification of the dideoxy chain termination protocol allowing direct

sequencing of PCR products (Winship, 1989), except that $MgCl_2$ and NaCl were added to the labelling mix only after DNA denaturation.

Additional methods related to recombinant DNA technology

Maniatis et al. (1982, 1989)

REFERENCES

Ahlquist, P., French, R., Janda, M. and Loesch-Fries, L.S. (1984) Proc. Natl. Acad. Sci. USA 81, 7066-7070

Auffray, C. and Rougeon, F. (1980) Eur. J. Biochem. 107, 303-314

Bellini, W.J., Englund, G., Rozenblatt, S., Arnheiter, H. and Richardson, C.D. (1985) J. Virol. 53, 908-919

Billeter, M.A. and Cattaneo, R. (1990) In Kingsbury, D. (ed), The Paramyxoviruses. Plenum Press, New York, in press

Burnstein, T., Jacobsen, L.B., Zeman, W. and Chen, T.T. (1974) Infect. Immun. 10, 1378-1382

Cattaneo, R., Will, H. and Schaller, H. (1984) EMBO J. 3, 2191-2196

Cattaneo, R., Rebmann, G., Schmid, A., Baczko, K., ter Meulen, V. and Billeter, M.A. (1987) EMBO J. 6, 681-688

Cattaneo, R., Schmid, A., Billeter, M.A., Sheppard, R.D. and Udem, S.A. (1988a) J. Virol. 62, 1388-1397

Cattaneo, R., Schmid, A., Eschle, D., Baczko, K., ter Meulen, V. and Billeter, M.A. (1988b) Cell 55, 255-265

Cattaneo, R., Kaelin, K., Baczko, K. and Billeter, M.A. (1989a) Cell 56, 759-764

Cattaneo, R., Schmid, A., Spielhofer, P., Kaelin, K., Baczko, K., ter Meulen, V., Pardowitz, J., Flanagan, S., Rima, B.K., Udem, S.A. and Billeter, M.A. (1989b) Virology 173, 415-425

Chattopadhyay, D. and Banerjee, A.K. (1987) Cell 49, 407-414

Cress, D.E, Kiefer, M.C. and Owens, R.A. (1983) Nucleic Acids Res. 11, 6821-6835

Dasmahapatra, B., Dasgupta, R., Saunders, K., Selling, B., Gallagher, T. and Kaesberg, P. (1986) Proc. Natl. Acad. Sci. USA 83, 63-66

Dawson, W.O., Beck, D.L., Knorr, D.A. and Grantham, G.L. (1986) Proc. Natl. Acad. Sci. USA 83, 1832-1836

Evans, D.J., Mc Keating, J., Meredith, J.M., Burke, K.L.,Katrak, K., John, A., Ferguson, M., Minor, P.D., Weiss, R.A., amd Almond, J.W. (1989) Nature 339, 385-389

Gilvarg, C., Jockusch, H. and Weissmann, C. (1975) Biochem. Biophys. Acta 414, 341-348

Gotoh, H., Shioda, T., Sakai, Y., Mizumoto, K. and Shibuta, H. (1989) Virology 171, 434-443

Luytjes, W., Krystal, M., Enami, M., Parvin, J.D., and Palese, P. (1989) Cell 59, 1107-1113.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York; also: 2nd edition: Sambrook, J., Fritsch, E.F., and Maniatis (1989)

Meier, E., Harmison, G.G. and Schubert, M. (1987) J. Virol. 61, 3133-3142

Meshi, T., Ishikawa, M, Motcyoshi, F., Semba, K. and Okada, Y. (1986) Proc. Natl. Acad. Sci. USA 83, 5043-5047

Niacin, E., Panicali, D., and Paoletti, E. (1982) Proc. Natl Acad. Sci. USA 79, 1593-1596

Racaniello V.R. and Baltimore D. (1981) Science 214, 916-919

Rager-Zisman, B., Egan, J.E., Kress, Y. and Bloom, B.R. (1984) J. Virol. 51, 845-855

Ray, J. and Fujinami, R.S. (1987) J. Virol. 61, 3381-3387

Rice, C.M., Levis, R., Strauss, J.H. and Huang, H.V. (1987) J. Virol. 61, 3809-3819

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487-491

Schmid, A., Cattaneo, R. and Billeter, M.A. (1987) Nucleic Acids Res. 15, 3987-3996

Sheppard, R.D., Raine, C,S., Bornstein, M.B., and Udem, S.A. (1986) Proc. Natl. Acad. Sci. USA 83, 7913-7917

Simon, A.E., Engel, H., Johnson, R.P. and Howell, S.H. (1988) EMBO J. 7, 2645-2651

Spaete, R.R., and Frenkel, N., (1982) Cell 30, 295-304

Spaete, R.R., and Mocarski, E.S. (1987) Proc. Natl. Acad. Sci. USA 84, 7213-7217

Tabler, M. and Sänger, H.L. (1985) EMBO J. 4, 2191-2199

Taniguchi, T., Palmieri, M. and Weissmann, C. (1978) Nature 274, 223-228

ter Meulen, V., Stephenson, J.R. and Kreth, H.W. (1983) Compr. Virol. 18, 105-159

Udem, S.A. (1984) J. Virol. Methods 8, 123-136

Weir P.J., Bajszar, G., and Moss, B. (1982) Proc. Natl. Acad. Sci. USA 79, 1210-1214

Whitt, M.A., Chong, L. and Rose, J.K. (1989) J. Virol. 63, 3569-3578

Winship, P.R. (1989) Nucleic Acids Res. 17, 1266

Xiong, C., Levis, R., Shen, P.,Schlesinger, S., Rice, C.M., and Huang, H.V. (1989) Science 243, 1188-1191

Claims

1. A cDNA molecule for the production of negative-strand RNA virus comprising
(a) the entire genomic or anti-genomic sequence of a non-segmented negative-strand RNA virus, operatively linked to
(b) an expression control sequence.

2. The cDNA molecule according to claim 1, wherein the expression control sequence (b) is an RNA polymerase promoter.

3. A vector containing a cDNA molecule according to claim 1 or 2.

4. The vector according to claim 3, containing an expressible DNA fragment which replaces a DNA region of said cDNA molecule or provides additional genetic information.

5. The vector according to claim 4, characterized in that the expressible DNA fragment is inserted into a region of said cDNA encoding a viral protein, said insertion being effected in a manner maintaining the reading frame and permitting the expression of said DNA fragment under the control of the signal sequences of said viral protein.

6. The vector according to claim 4, characterized in that the expressible DNA fragment is inserted into a non-coding region of said cDNA and flanked by viral signal sequences or heterologous signal sequences controlling the expression of said DNA fragment.

7. The vector according to any one of claims 3 to 6, which is capable of replicating in a prokaryotic host.

8. The vector according to any one of claims 3 to 6 which is capable of replicating in a eukaryotic host.

9. The vector according to any one of claims 3 to 8, wherein said expressible DNA fragment is a DNA fragment being homologous or heterologous with respect to the negative-strand RNA virus and encoding at least one immunogenic epitope.

10. The vector according to claim 9, wherein said expressible DNA fragment encodes at least one immunogenic epitope of a pathogen.

11. The vector according to claim 10, wherein said expressible DNA fragment is derived from a virus, a bacterium, or a parasite.

12. The vector according to any one of claims 3 to 11, wherein said expressible DNA fragment encodes an immunogenic epitope being able to elicit a protective immune response.

13. A prokaryotic or eukaryotic host cell transformed with a vector according to any one of claims 3 to 12.

14. A helper cell containing a vector according to any one of claims 3 to 12, said helper cell providing the viral proteins necessary for encapsidation, transcription and replication of RNA resulting from transcription of the DNA contained in the vector, and allowing the assembly of infectious negative-strand RNA viruses.

15. An infectious negative-strand RNA virus strain isolated from the helper cell of claim 14.

16. A method for the preparation of recombinant vaccine strains of negative-strand RNA virvses, comprising the steps of:
(a) introducing a recombinant vector according to any one of claims 3 to 12 as a complex with an RNA polymerase into competent helper cells producing the viral proteins required for the assembly of infectious virus of the negative-strand RNA virus; and
(b) recovering the assembled infectious negative-strand RNA viruses.

17. The method according to claim 16, wherein said r combinant vector is obtainable by a method

comprising the following steps:

(A) preparing a full-length genomic cDNA molecule by assembly of cDNA clones derived from mono- and/or polycistronic viral mRNA;

(B) inserting said cDNA of step (a) into a vector under the control of an expression control sequence, preferably an RNA polymerase promoter, so as to allow the initiation of transcription of said cDNA by said control sequence;

(C) incorporating into a viral protein-encoding region of said cDNA a DNA fragment in reading frame so as to be expressible under the control of the signal sequences of said viral protein or incorporating said DNA fragment into a non-coding region of said cDNA so as to be expressible under the control of a flanking viral or heterologous signal sequence, wherein said expressible DNA fragment replaces a DNA region of said cDNA molecule or provides additional genetic information;

(D) cloning the obtained recombinant vector in a microorganism; and

(E) isolating said recombinant vector.

18. Vaccine containing an infectious negative-strand RNA virus strain according to claim 15 or obtainable by the method according to claim 16 or 17, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

Scheme for cDNA cloning of negative-strand RNA viral genomes and expression of the cDNA as replicating virus

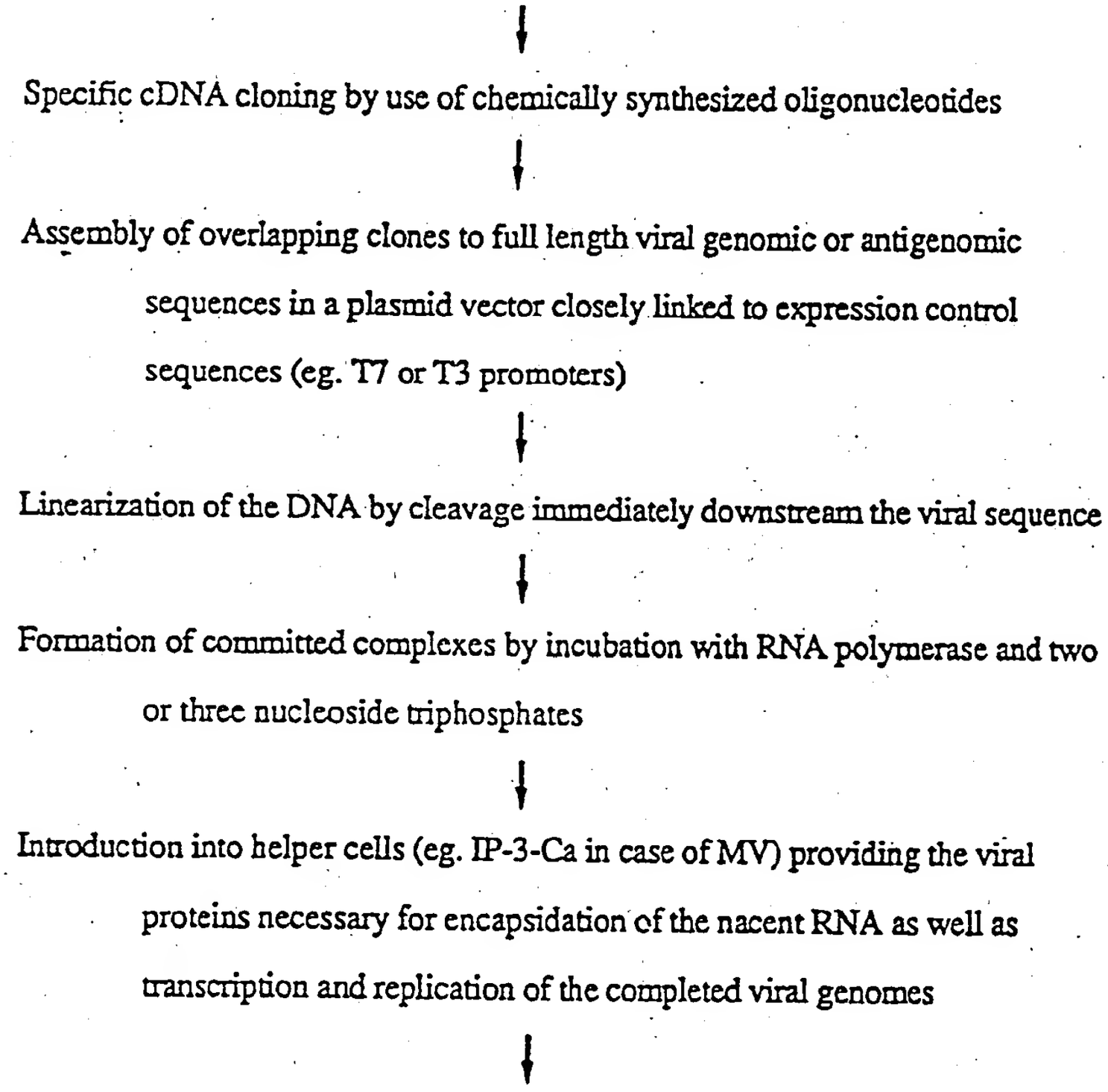

Figure 1

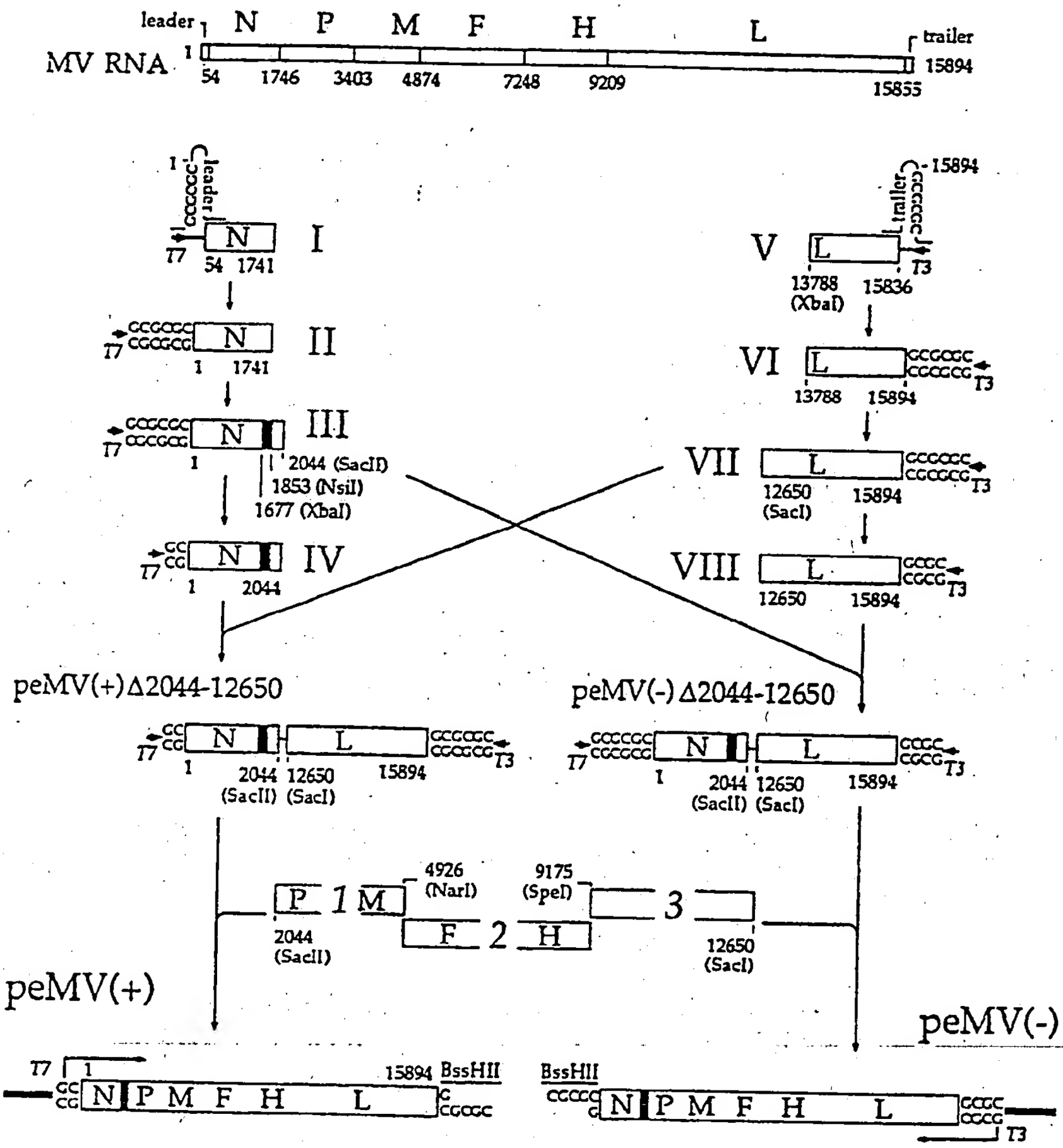
leader
N
P
M
F
H
L
trailer
MV RNA
1
54
1746
3403
4874
7248
9209
15855
15894
I
II
III
IV
V
VI
VII
VIII
peMV(+)Δ2044-12650
peMV(-)Δ2044-12650
peMV(+)
peMV(-)
T7
T3
2044 (SacII)
1853 (NsiI)
1677 (XbaI)
13788 (XbaI)
15836
12650 (SacI)
15894
4926 (NarI)
9175 (SpeI)
BssHII

Figure 2

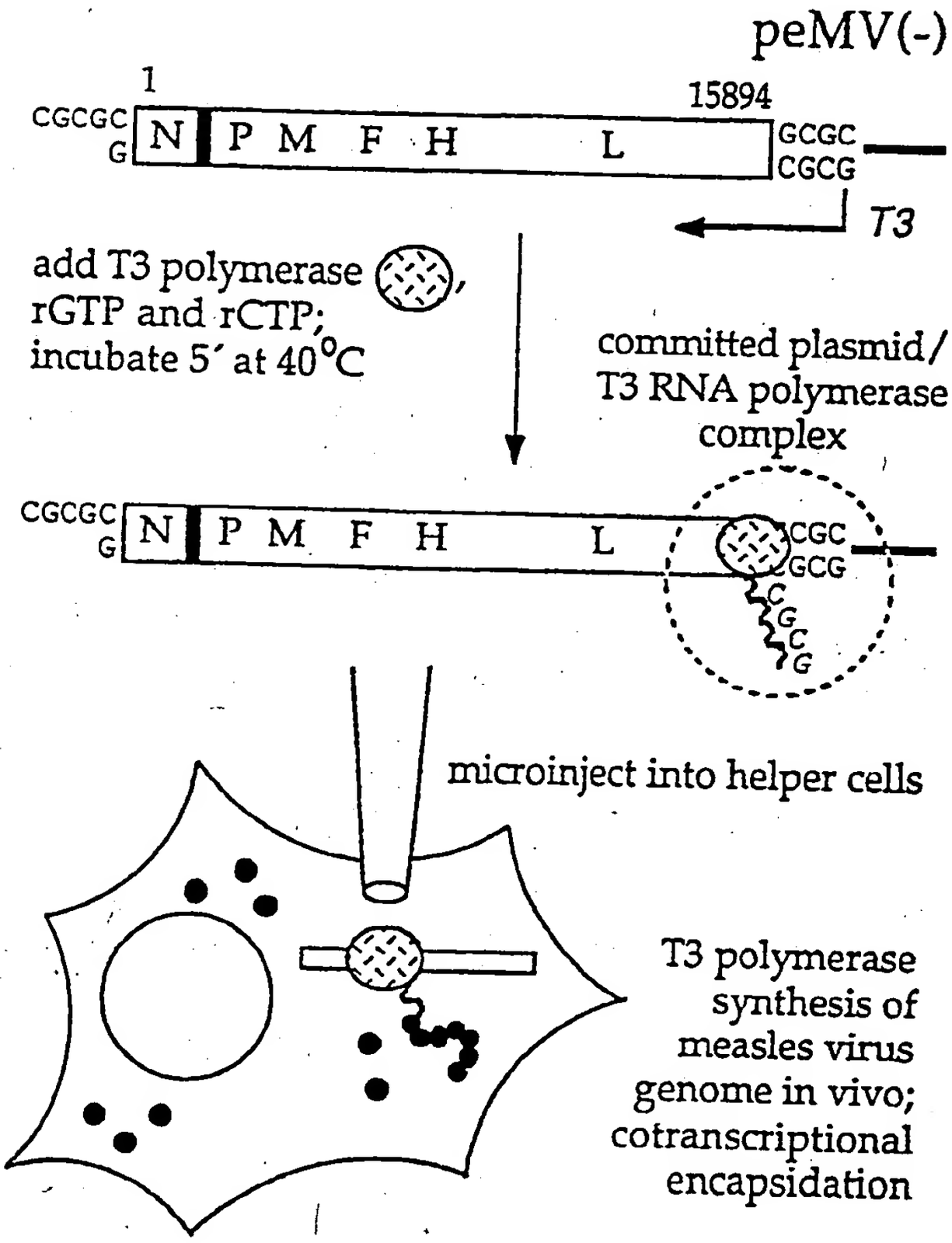
peMV(-)
1
15894
CGCGC
G
N
P M F H L
GCGC
CGCG
T3
add T3 polymerase,
rGTP and rCTP;
incubate 5′ at 40°C
committed plasmid/
T3 RNA polymerase
complex
CGCGC
G
N
P M F H L
CGC
GCG
C
G
C
G
microinject into helper cells
T3 polymerase
synthesis of
measles virus
genome in vivo;
cotranscriptional
encapsidation

Figure 3

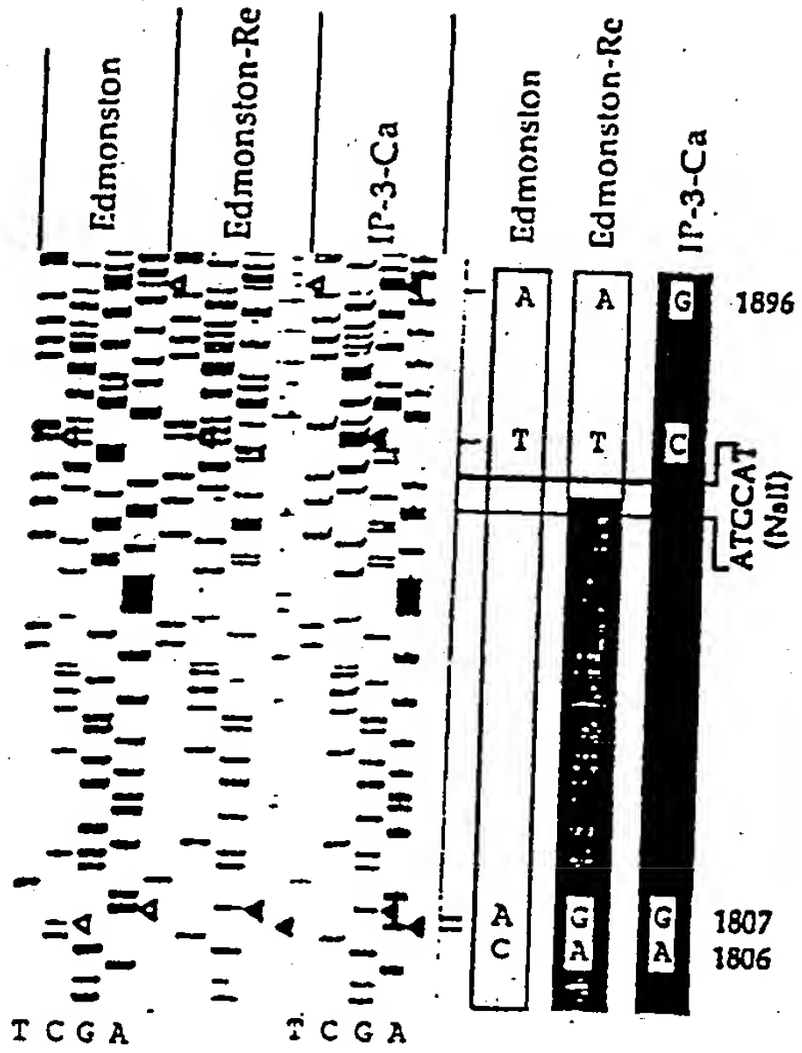
Edmonston
Edmonston-Re
IP-3-Ca
Edmonston
Edmonston-Re
IP-3-Ca
A
T
A
T
G
C
1896
ATGCAT
(NsiI)
A
C
G
A
G
A
1807
1806
T C G A
T C G A

Figure 4

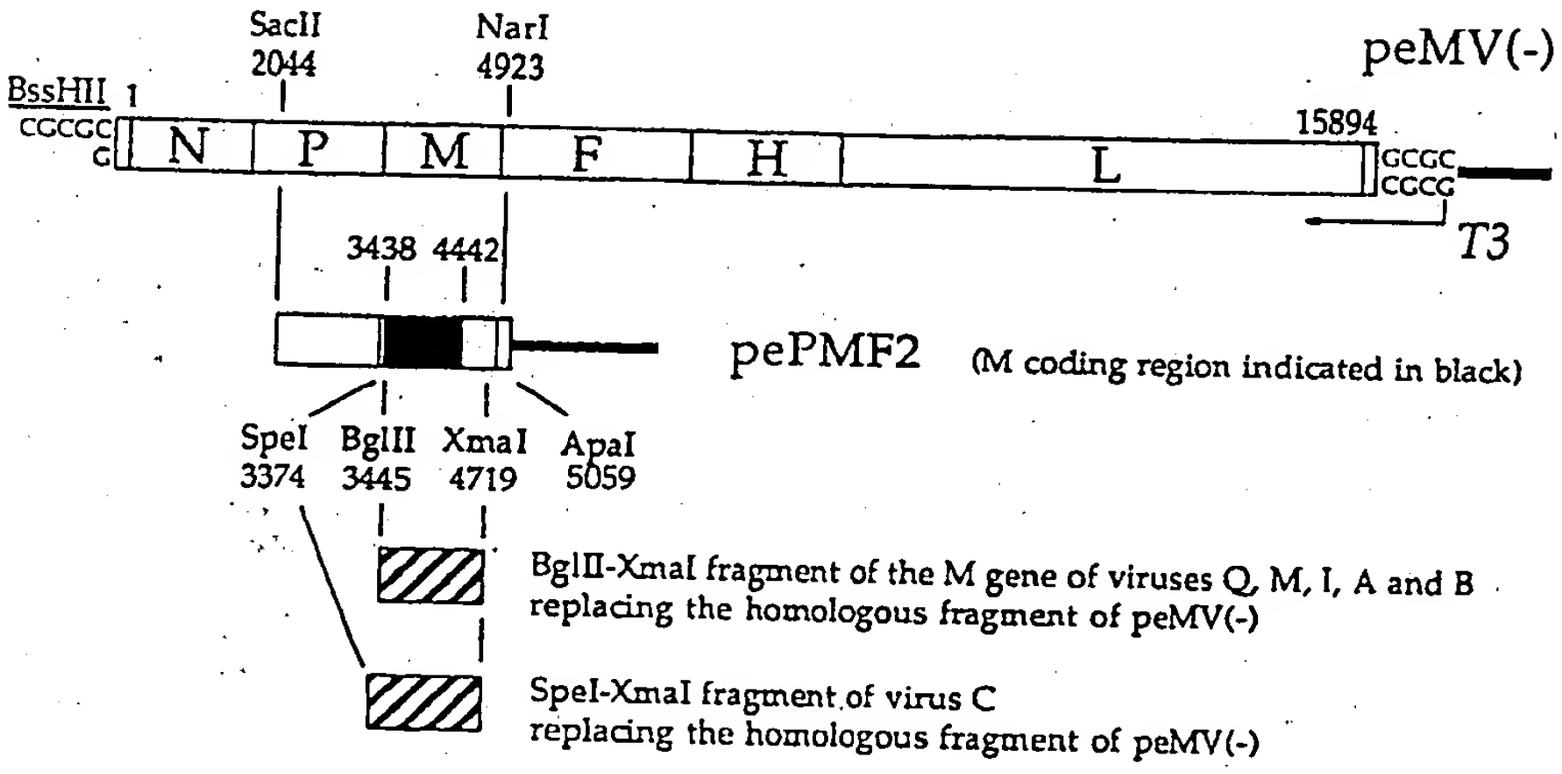
SacII
2044
NarI
4923
peMV(-)
BssHII 1
CGCGC
G
N
P
M
F
H
L
15894
GCGC
CGCG
T3
3438 4442
pePMF2
(M coding region indicated in black)
SpeI
3374
BglII
3445
XmaI
4719
ApaI
5059
BglII-XmaI fragment of the M gene of viruses Q, M, I, A and B
replacing the homologous fragment of peMV(-)
SpeI-XmaI fragment of virus C
replacing the homologous fragment of peMV(-)

Figure 5

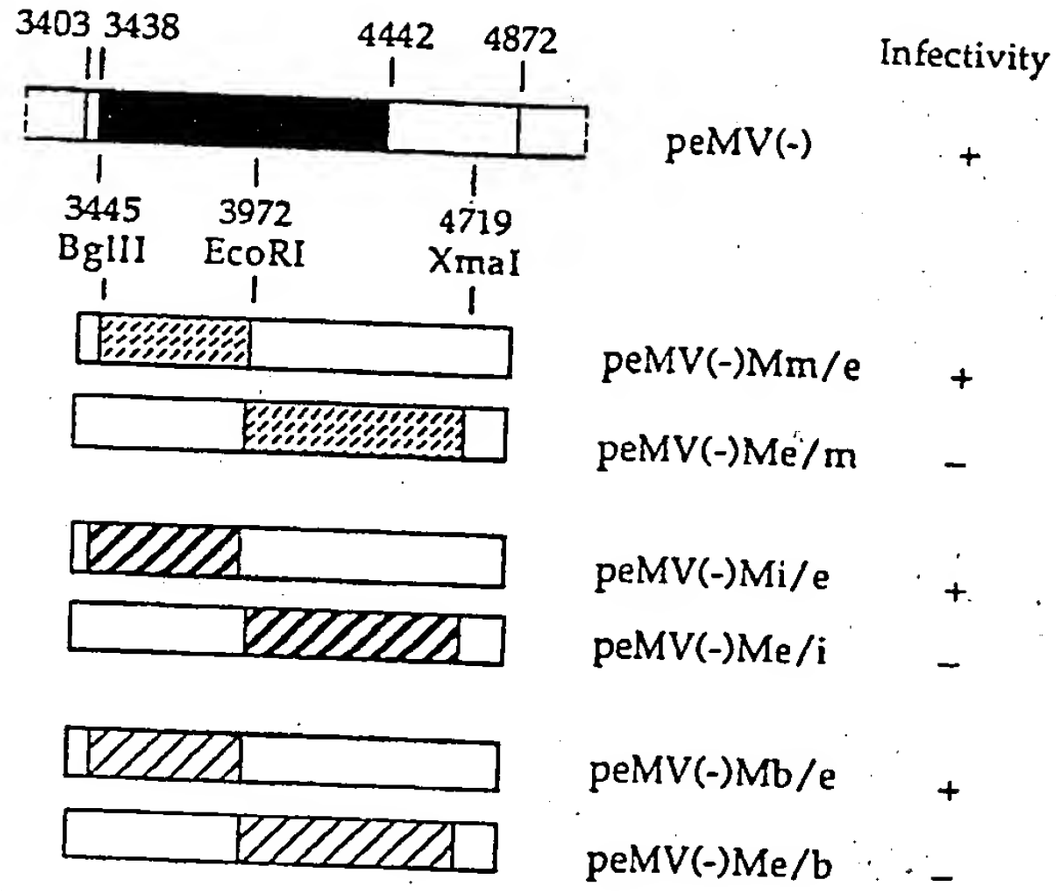
3403
3438
4442
4872
Infectivity
peMV(-)
+
3445
BglII
3972
EcoRI
4719
XmaI
peMV(-)Mm/e
+
peMV(-)Me/m
–
peMV(-)Mi/e
+
peMV(-)Me/i
–
peMV(-)Mb/e
+
peMV(-)Me/b
–

Figure 6

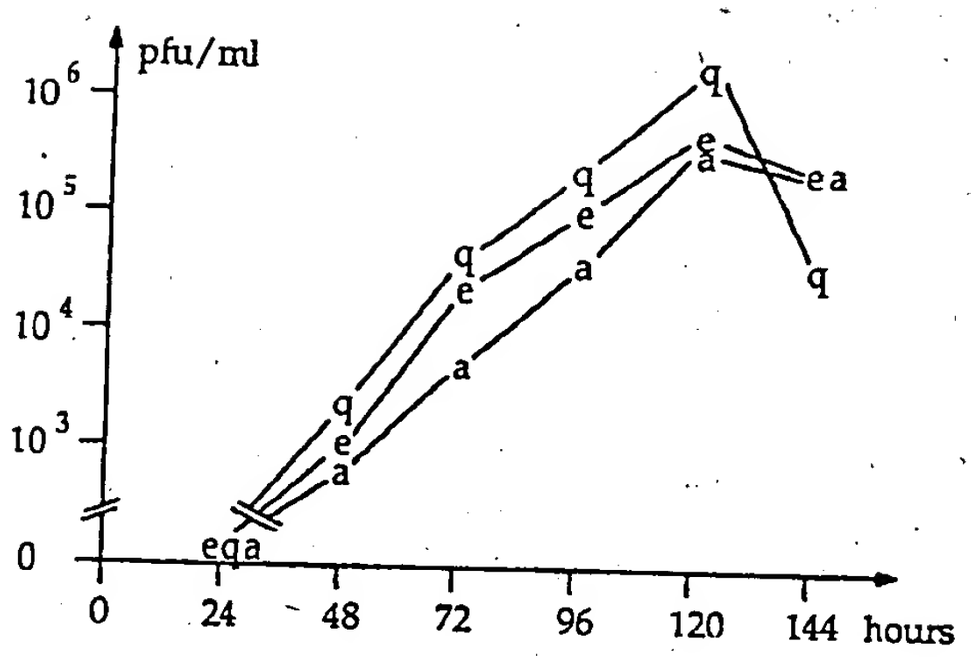

Figure 7

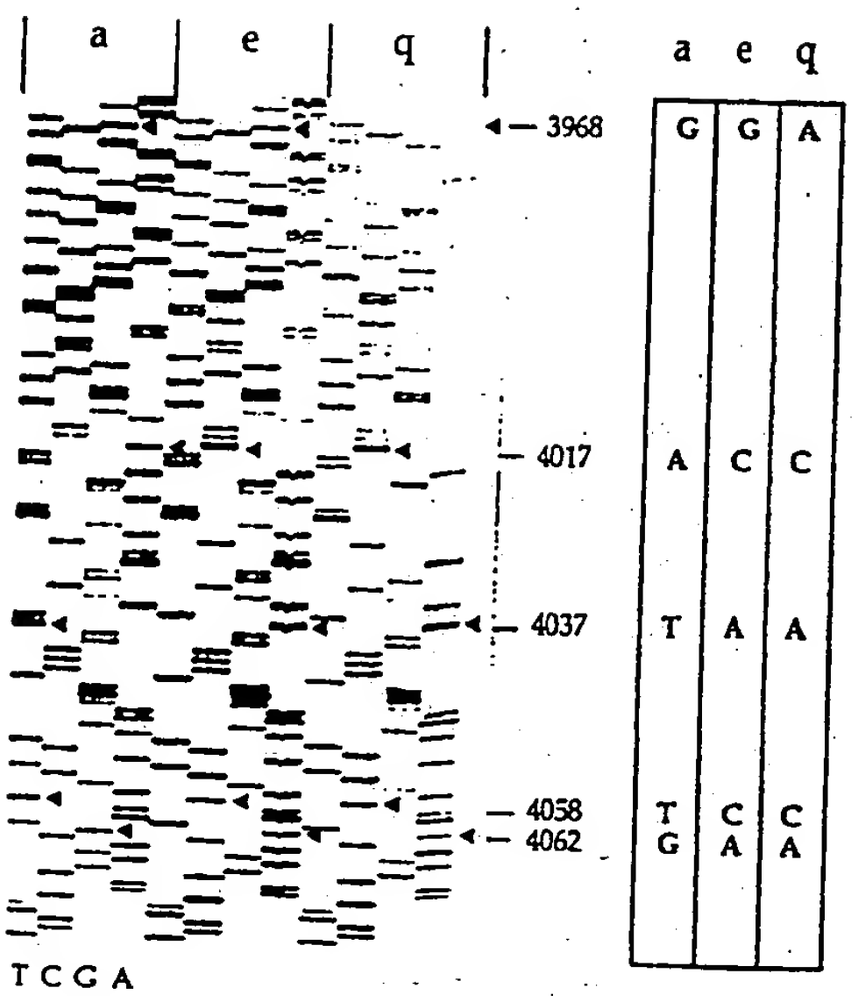

Figure 8

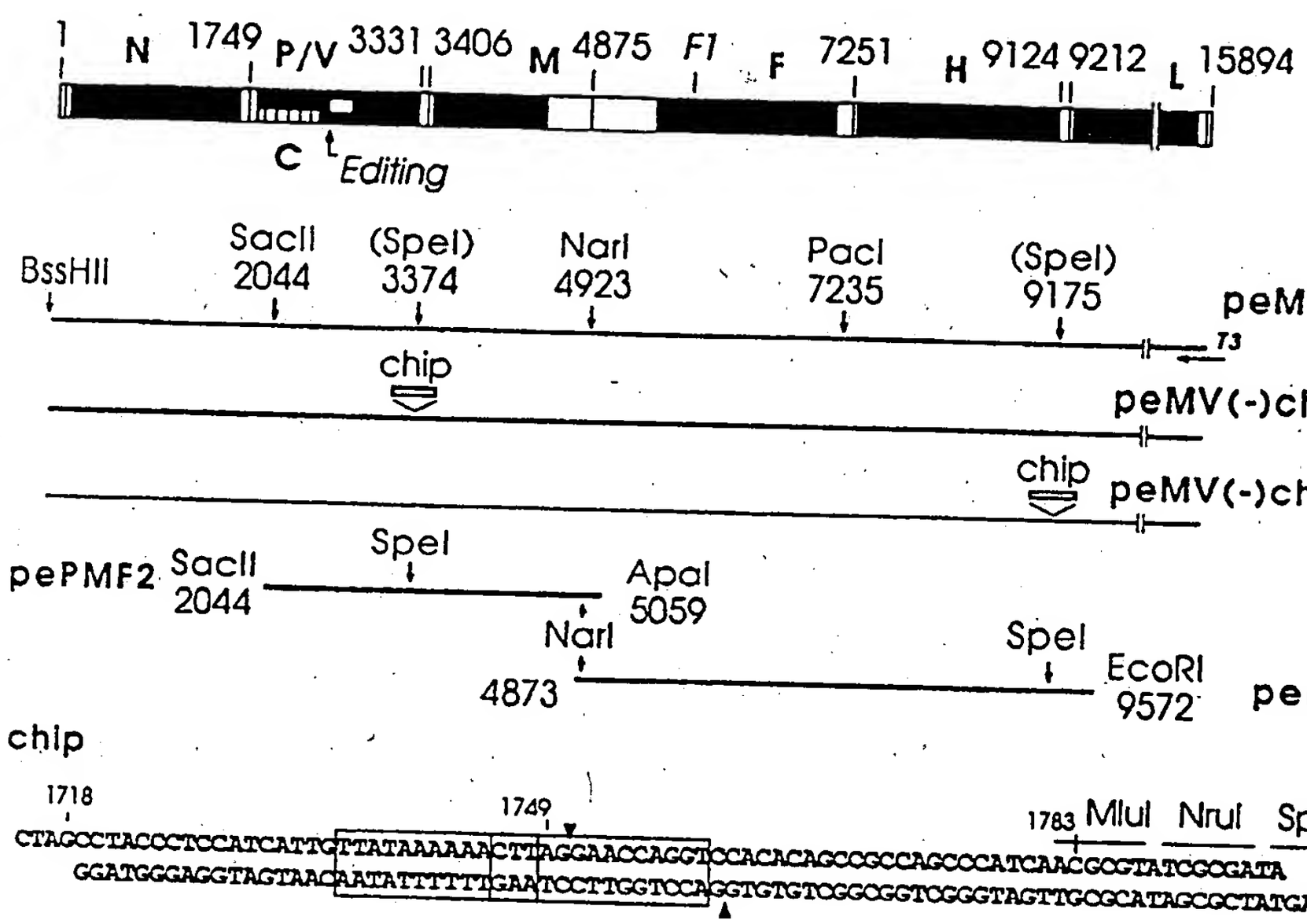
1
N
1749
P/V
3331
3406
M
4875
F1
F
7251
H
9124
9212
L
15894
C
Editing
BssHII
SacII 2044
(SpeI) 3374
NarI 4923
PacI 7235
(SpeI) 9175
peMV(-)
T3
chip
peMV(-)chipP
chip
peMV(-)chipH
pePMF2
SacII 2044
SpeI
ApaI 5059
NarI 4873
SpeI
EcoRI 9572
peFHL
chip
1718
1749
1783
MluI
NruI
SpeI
CTAGCCTACCCTCCATCATTGTTATAAAAAACTTAGGAACCAGGTCCACACAGCCGCCAGCCCATCAACGCGTATCGCGATA
GGATGGGAGGTAGTAACAATATTTTTTGAATCCTTGGTCCAGGTGTGTCGGCGGTCGGGTAGTTGCGCATAGCGCTATGATC

Figure 9

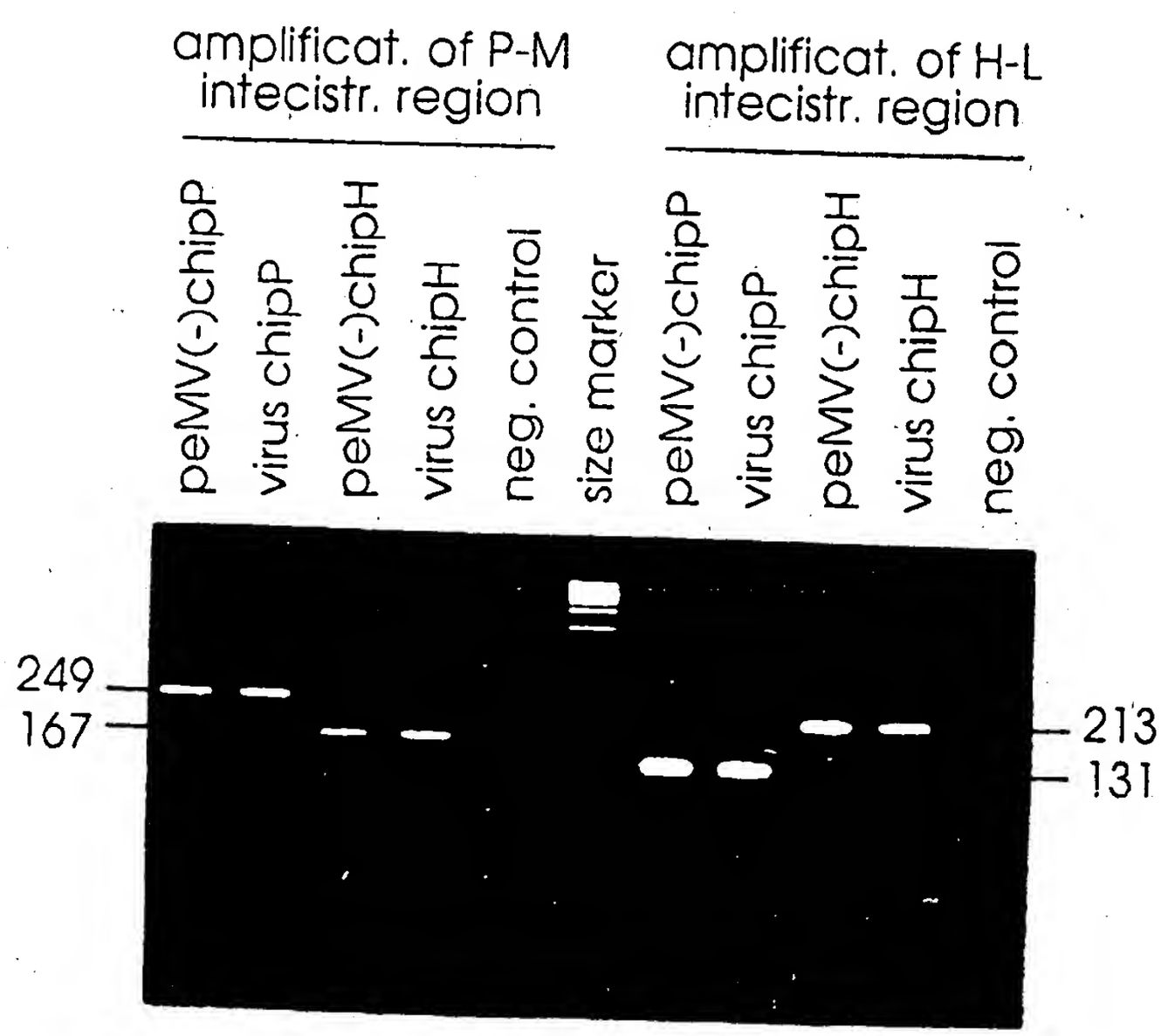
amplificat. of P-M intecistr. region
amplificat. of H-L intecistr. region
peMV(-)chipP
virus chipP
peMV(-)chipH
virus chipH
neg. control
size marker
peMV(-)chipP
virus chipP
peMV(-)chipH
virus chipH
neg. control
249
167
213
131

Figure 10

European Patent Offic

EUROPEAN SEARCH REPORT

Application Number

EP 91 10 1278

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X,D	NUCLEIC ACIDS RESEARCH, vol. 15, no. 10, 1987, pages 3987-3996, IRL Press Ltd, Oxford, GB; A. SCHMID et al.: "A procedure for selective full length cDNA cloning of specific RNA species * Whole document, especially figure 1; page 3995, last paragraph before Acknowledgments *	1-13	C 12 N 15/45 C 12 N 15/47 C 12 N 5/10 A 61 K 39/165 A 61 K 39/205 C 12 N 7/00
A	EP-A-0 237 686 (INSTITUT PASTEUR)(23-09-1987) * Whole document *	1-18	
P,X	THE EMBO JOURNAL, vol. 9, no. 2, February 1990, pages 379-384, Oxford University Press, Eynsham, Oxford, GB; I. BALLART et al.: "Infectious measles virus from cloned cDNA"	1-18	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)
C 07 K C 12 N A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
The Hague	08 May 91	CUPIDO M.

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same catagory
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention

E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document